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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> MONOCLONAL ANTIBODIES TO FIBRIN  <b>(57) Abstract</b> <p>Monoclonal antibodies specific for fibrin, its plasma precursor fibrinogen and their plasmin derived degradation products. Monoclonal antibodies capable of reacting with fibrin, fibrinogen and their degradation products are of value for the diagnosis and treatment of vascular disorders, such as thrombosis and disseminated intravascular coagulation. The monoclonal antibodies of this invention possess distinctive characteristics and capabilities which make them suitable for <i>in vitro</i> clinical diagnostic purposes, such as their ability to distinguish between fibrinogen and fibrin and their derivatives. Moreover, the monoclonal antibodies of this invention allow the accurate measurement of the <i>in vivo</i> concentration of these blood proteins.</p>		

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## MONOCLONAL ANTIBODIES TO FIBRIN

1. INTRODUCTION

This invention relates to monoclonal antibodies specific for fibrin, its plasma precursor fibrinogen and their plasmin derived degradation products. Monoclonal antibodies capable of reacting with fibrin, fibrinogen and their degradation products are of value for the diagnosis and treatment of vascular disorders, such as thrombosis and disseminated intravascular coagulation. The monoclonal antibodies of this invention possess distinctive characteristics and capabilities which make them suitable for in vitro clinical diagnostic purposes, such as their ability to distinguish between fibrinogen and fibrin and their derivatives. Moreover, the monoclonal antibodies of this invention allow the accurate measurement of the in vivo concentration of these blood proteins.

The invention also provides methods for production of the monoclonal antibodies by hybridoma techniques. Once cloned, all lines can be maintained continuously to produce, at high titer, an unlimited and homogeneous monoclonal antibody population that can be isolated and/or purified and used clinically for in vitro diagnostic assays. In addition, these monoclonal antibodies are advantageously directed against individual antigenic epitopes, thus, making the findings based upon their applications reliable and accurate.

~~The monoclonal antibodies of this invention may also~~  
be used in a variety of in vivo therapeutic or diagnostic applications including carriers for fibrinolytic agents and as reagents for imaging an intravascular thrombus.

## 2. BACKGROUND OF THE INVENTION

### 2.1. MONOCLONAL ANTIBODIES

Kohler and Milstein are generally credited with having  
5 devised the techniques that successfully resulted in the  
formation of the first monoclonal antibody-producing  
hybridomas (G. Kohler and C. Milstein, 1975, Nature 256:495-  
497; 1976, Eur. J. Immunol. 6:511-519). By fusing antibody-  
forming cells (spleen lymphocytes) with myeloma cells  
10 (malignant cells of bone marrow primary tumors) they created  
a hybrid cell line, arising from a single fused cell hybrid  
(called a hybridoma or 'clone') which had inherited certain  
characteristics of both the lymphocytes and myeloma cell  
lines. Like the lymphocytes (taken from animals primed with  
15 sheep red blood cells as antigen), the hybridomas secreted a  
single type of immunoglobulin specific to the antigen;  
moreover, like the myeloma cells, the hybrid cells had the  
potential for indefinite cell division. The combination of  
these two features offered distinct advantages over  
20 conventional antisera. Whereas antisera derived from  
vaccinated animals are variable mixtures of polyclonal  
antibodies which never can be reproduced identically,  
monoclonal antibodies are highly specific immunoglobulins of  
a single type. The single type of immunoglobulin secreted by  
25 a hybridoma is specific to one and only one antigenic  
determinant, or epitope, on the antigen, a complex molecule  
having a multiplicity of antigenic determinants. For  
instance, if the antigen is a protein, an antigenic  
determinant may be one of the many peptide sequences  
30 (generally 6-7 amino acids in length; M.Z. Atassi, 1980,  
Molec. Cell. Biochem. 32: 21-43) within the entire protein  
molecule. Hence, monoclonal antibodies raised against a  
single antigen may be distinct from each other depending on  
the determinant that induced their formation; but for any  
35 given clone, all of the antibodies it produces are identical.

Furthermore, the hybridoma cell line is easily propagated in vitro or in vivo, and yields monoclonal antibodies in extremely high concentration.

## 2.2. HEMOSTATIC MECHANISMS

5 Generally stated, blood coagulation is the principal hemostatic defense in humans. The transformation of blood into a clot involves among other factors the conversion of the plasma protein fibrinogen to fibrin. Fibrinogen is a  
10 soluble and rod-like protein typically present in the human plasma. It is produced by the liver and has a molecular weight of about 340,000 Daltons. The formation of fibrin from fibrinogen is a thrombin-dependent reaction. Thrombin, an enzyme whose production is typically triggered by vessel  
15 damage, catalyzes the conversion of fibrinogen to fibrin by proteolytically cleaving fibrinopeptides, A and B from the A alpha, and B Beta chains, respectively. The resultant soluble fibrin monomers noncovalently polymerize. In the presence of factor XIII (fibrin stabilizing factor) and  
20 calcium, the gel is transformed into a nonsoluble fibrin clot.

The fibrinolytic system is the body's means for eliminating clots. This system becomes activated at the completion of the coagulation process. A protein known as plasminogen, which circulates in the blood system, may become activated to form the proteolytic enzyme plasmin. Plasmin is able to degrade fibrin and, thereby, dissolve a clot. When fibrinogen is exposed to plasmin, it breaks down first into fragments x and y and after further exposure, into smaller fragments D and E.

Fibrinogen and fibrin degradation products (FDP) generally refer to fragments generated during fibrinolysis as a result of the proteolytic effect of plasmin on fibrinogen and fibrin, respectively. These fragments include the

intermediate fragments X and Y resulting from fibrinogen degradation. Further plasmin digestion of Fragment Y generates the fibrinogen degradation products, fragments D and E. Fragments derived from plasmin digestion of fibrin consists of fragments X, Y, D and E from noncrosslinked fibrin, and crosslinked D-dimer, E complex, Y dimer, Y-D, D-dimer, and X oligomer from crosslinked fibrin.

Small amounts of FDP, particularly from fibrin degradation, are present in normal serum but are markedly elevated in the serum of patients with thromboembolic conditions such as myocardial infarction, deep vein thrombosis, pulmonary emboli and disseminated intravascular coagulation (DIC). The occurrence of elevated fibrinogen degradation products in serum is rare and most commonly occurs during fibrinolytic therapy when plasmin levels are greatly elevated. Therefore, blood levels of these products can be useful in assessing vascular disorders.

It has been hypothesized that formation of clots within intact vessels (as might be the case with the above disease states) may be caused by a hyperactive clotting mechanism or that minor changes to vessel walls trigger the coagulation cascade. These changes could be related to decreased blood flow and oxygen supply to the blood vessel walls, arteriosclerosis and inflammation of the vessels caused by infection or other agents. Clots which form could grow and block an entire vessel resulting in damage to other tissue. Therefore, it is clear that a method to detect developing thrombus *in vivo* and FDPs *in vitro* prior to extensive clot formation could be life-saving.

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### 2.3. ASSAYS USING ANTIBODIES TO DETECT FIBRINOGEN DERIVATIVES

Due to the importance of measuring the in vivo concentrations of fibrin, fibrinogen and its derivatives in the diagnosis and treatment of impaired hemostasis, much effort has gone into developing specific and accurate analytical methods for their determination. Many of the diagnostic methods devised are based on the production of polyclonal antisera to fibrin and fibrinogen derivatives. Although there have been reports of specific antisera, the inherent problem of antiserum to fibrin and fibrinogen derivatives remains to be the lack of specificity due to the relatively small number of neoantigenic sites generated in fibrin and FDPs.

The development of hybridoma technology has made available a new diagnostic tool in the form of monoclonal antibodies which can be directed at individual antigenic sites. This technology allows for the generation of highly specific analytical probes. The production of antibodies specific for antigenic sites on fibrin, fibrinogen and their derivatives is still complicated by the high level of conservation of the covalent structure of fibrinogen when it is converted to fibrin and its degradation products. There have been several recent reports of fibrin specific monoclonal antibodies, and the strategy employed was based on the hypothesis that antigenic sites are exposed on the amino terminus of fibrinogen when the fibrinopeptides are cleaved by thrombin. A monoclonal antibody was generated to human fibrin II. The immunogen consisted of the NH<sub>2</sub>-terminal cyanogen bromide fragments of human fibrin II. The antibody recognizes the new N-terminus of the B beta chain after fibrinopeptide B is released (see Kudryk, B. et al. Mol.



Immunology Vol. 21 No. 1 pp. 89-94.) In this reference, the peptide was generated from the native fibrin molecule and not actively synthesized.

There is further need for monoclonal antibodies which are able to differentiate between fibrinogen and fibrin and their derivatives, for use in in vitro and in vivo diagnostic and therapeutic applications. It is an object of this invention to provide such monoclonal antibodies.

### 10 3. SUMMARY OF THE INVENTION

Prior to the present invention, there has been no report of monoclonal antibodies generated to soluble fibrin which could distinguish between fibrinogen and fibrin and which also had a delaying effect on the dissolution of clots by tissue plasminogen activator in vivo. The monoclonal antibodies of this invention, therefore, provide a new means for the in vitro or in vivo diagnosis and/or treatment of vascular disorders and further, provide a new means for in vivo regulation of certain aspects of the blood coagulation/fibrinolysis systems.

20 In a preferred embodiment, the present invention provides a method for producing monoclonal antibodies against both fibrinogen and fibrin and/or their degradation products. The monoclonal antibodies of this invention can be used immunologically to distinguish between the presence of fibrinogen or fibrin in an in vitro sample by virtue of the different specificity of each antibody for different antigenic sites. Hence, the monoclonal antibodies of the present invention represent new in vitro immunodiagnostic reagents for the early and accurate detection of certain vascular diseases.

In addition to their use as in vitro immunodiagnostic reagents for vascular disease, the monoclonal antibodies of the present invention can also be used diagnostically in vivo. The capability of these antibodies to bind to fibrin

would enable the localization of fibrin clotting in vivo when said monoclonal antibodies are appropriately labeled by, for instance, radioactive compounds or magnetic probes.

The monoclonal antibodies of the present invention may also be used to monitor the course of fibrinolytic therapy. The concentration or level of FDP present in vivo may be monitored by an in vitro radio-immuno or enzyme-linked diagnostic assay using the monoclonal antibodies of this invention which are directed against fibrinogen and fibrinogen degradation products. As fibrinolytic therapy progresses, i.e., after treatment with tPA, the FDP levels would increase in the blood plasma. This increase would be detectable and measurable using the monoclonal antibodies of this invention.

An additional aspect of the present invention is the use of the monoclonal antibodies as "carriers" of desired reagents to fibrin clots or other areas of fibrin concentration. The substances carried by the monoclonal antibodies may be directed at a clot to speed its dissolution or they could be targeted towards tissue or conditions surrounded by the fibrin associated clot in vivo (i.e., cytotoxic or chemotherapeutic agents). These substances may be bound to the monoclonal antibodies in a variety of ways including covalent attachment.

Because the monoclonal antibodies are produced by hybridoma techniques, the present invention provides ~~theoretically immortal cell lines capable of consistently~~ producing high titers of single specific antibodies against a distinct epitope on fibrinogen or fibrin or their proteolytic fragments. This is a distinct advantage over the traditional technique of raising antibodies in immunized animals where the resulting sera contain multiple antibodies of different specificities that vary in both type and titer with each animal, and, in individual animals, with each immunization. Thus of soluble fibrin also provides an advantage ver

immunization with synthetic peptides representing the new amino terminus generated after thrombin cleavage of fibrinogen, since additional and different epitopes are presented as potential antigens for generation of the monoclonal antibodies.

#### 4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 graphically illustrates the determination of binding constants of monoclonal antibody, DG1, to fibrin (A) and fibrinogen (B), using a modified Scatchard plot. The ratio of the concentration of bound antibody  $[Ab]_B$  to free  $[Ab]_F$ , is plotted against  $[Ab]_B$ .

FIGURE 2 graphically illustrates the competitive binding of fibrinogen and fibrin for antibodies, DG1 ( $\blacklozenge$ ) and DG2 ( $\blacklozenge$ ). A subsaturating level of  $^{125}\text{I}$ -labelled antibody was added to fibrin coated wells, containing various levels of fibrinogen. The 96-well plates were incubated at  $37^\circ\text{C}$  for 90 minutes. After extensive washing with PBS Tween, the amount of antibody bound to fibrin in the individual wells was measured in a gamma counter. The results are the means of triplicate experiments.

FIGURE 3 graphically illustrates a solution phase competition assay between fibrin and fibrinogen for monoclonal antibodies DG1 ( $\circ$ ) and DG2 ( $\square$ ). A crosslinked fibrin clot was sonicated to produce a homogeneous fibrin suspension. Aliquots of the fibrin suspension were mixed with a range of fibrinogen solution. A constant subsaturating level of  $^{125}\text{I}$ -labelled monoclonal antibody was added to the mixture incubated at  $37^\circ\text{C}$  for 1 hour. The fibrin clot was recovered by centrifugation and washed extensively with PBS Tween. The amount of antibody bound to the fibrin clot was determined by measuring the  $^{125}\text{I}$  activity.  $^{125}\text{I}$ -labelled BSA ( $\blacklozenge$ ) was used in a control experiment to determine the extent of protein entrapment during centrifugation of the fibrin clot.

FIGURE 4 graphically illustrates a determination of the effect of monoclonal antibody, bound to fibrin, on the activity of t-PA. Aliquots of monoclonal antibody solution (0.2 ml) were incubated in fibrin coated wells of a 96-well microtiter plate for 90 minutes at 37°C. The wells were extensively washed with PBS Tween and a chromogenic assay was performed, to determine the t-PA activity, as described in the examples section. A rate assay was performed by measuring the change in absorbance at 405 nm ( $DA_{405}$ ) at different time points. The rate of production of pNA, in the presence of various concentrations of antibody, was determined from the slope of the line obtained by plotting  $DA_{405}$  against  $T^2$ . (A) represents the effect observed when antibody DG1 was bound to fibrin and (B) represents the effect of antibody DG2.

FIGURE 5 graphically illustrates the rate of tPA-mediated plasmin generation, using a chromogenic substrate, S-2251, in the presence of monoclonal antibodies DG1 ( $\diamond-\diamond$ ) and DG2 ( $\blacklozenge$ ).

FIGURE 6 graphically illustrates the effect of monoclonal antibody DG1 on thrombolysis using an animal model system.  $^{125}I$ -labelled fibrinogen (10 ug, 15 uCi in 0.5 ml saline) was injected intravenously (i.v.) at  $t=0$ . Thrombosis was induced by i.v. infusion of thrombin starting at  $t=30$  minutes. The  $^{125}I$  activity was measured over the lung field, for 1 minutes periods at 5 minute intervals. Monoclonal antibody DG1 [0.2 nmoles ( $\blacksquare-\blacksquare$ ) and 1 nmole ( $\circ-\circ$ )] was injected, i.v., at  $t=15$  minutes. In the control experiment ( $\blacklozenge$ ) no antibody was injected.

## 5. DESCRIPTION OF THE INVENTION

### 5.1. THE ANTIGEN

In the embodiment of the invention described herein,  
5 monoclonal antibodies are raised against human fibrin. This  
protein is found naturally in the human blood stream. The  
following procedure was used to prepare the mixture of  
soluble and insoluble fibrin used as an antigen for  
immunization. Human fibrinogen (Kabi-Sweden) 26 mg dissolved  
10 in 1 ml phosphate buffered saline pH 7.4, was passed over a  
Sephacrose lysine column to remove contaminating plasminogen.  
The fibrinogen solution was then incubated at room  
temperature with thrombin (Miles Laboratories), 2 NIH units,  
units/ml until clot formation occurred. Soluble fibrin was  
15 prepared by physical disruption of the clot in 4 M urea. The  
antigen preferred in this invention may comprise soluble  
fibrin, insoluble fibrin or a mixture of both soluble and  
insoluble fibrin with the mixture being the most preferred  
form.

### 5.2. SOMATIC CELLS

20 Somatic cells with the potential for producing  
antibody and, in particular B lymphocytes, are suitable for  
fusion with a B-cell myeloma line. Those antibody-producing  
cells that are in the dividing plasmablast stage fuse  
25 preferentially. Somatic cells may be derived from the lymph  
nodes, spleens and peripheral blood of primed animals, and  
the lymphatic cells of choice depend to a large extent on  
their empirical usefulness in the particular fusion system.  
Once-primed or hyperimmunized, animals can be used as a  
30 source of antibody-producing lymphocytes. Mouse lymphocytes  
give a higher percentage of stable fusions with the mouse  
myeloma lines described in Section 5.3. However, the use of  
rat or rabbit cells is also possible.

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Alternatively, human somatic cells capable of producing antibody, specifically, B lymphocytes, are suitable for fusion with myeloma cell lines. While B lymphocytes from biopsied spleens or lymph nodes of individuals may be used, the more easily accessible peripheral blood B lymphocytes are preferred. The lymphocytes may be derived from patients with diagnosed breast carcinomas or adenocarcinomas.

### 5.3. MYELOMA CELLS

Specialized myeloma cell lines have been developed from lymphocyte tumors for use in hybridoma-producing fusion procedures (G. Kohler and C. Milstein, 1976, Eur. J. Immunol. 6:511-519; M. Schulman *et al.*, 1978, Nature 276:269-270). The cell lines have been developed for at least three reasons. The first reason is to facilitate the selection of fused myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocyte tumor cells to produce their own antibodies. The purpose of using monoclonal techniques is to obtain immortal fused hybrid cell lines that produce the desired single specific antibody genetically directed by the somatic cell component of the hybridoma. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing light or heavy immunoglobulin chains or those deficient in antibody secretion mechanisms are used. A third reason for selection of these cell lines is their suitability and efficiency for fusion.

Several myeloma cell lines may be used for the production of fused cell hybrids, including NS-1, X63-Ag8, NIS-Ag4/1, MPC11-45.6TG1.7, X63-Ag8.653, Sp2/0-Ag14, FO, and S194/5XX0.Bu.1., all derived from mice, 210-.RCY3.Ag1.2.3 derived from rats, and U-226AR and GM1500GTGAL<sub>2</sub>, derived from

humans. (G.J. Hammerling, U. Hammerling and J.F. Kearney, eds., 1981, Monoclonal antibodies and T-cell hybridomas In J.L. Turk, ed. Research Monographs in Immunology, Vol. 3, Elsevier/North Holland Biomedical Press, New York).

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#### 5.4. FUSION

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a proportion which may vary from about 20:1 to about 1:1 in the presence of an agent or agents (chemical, viral or electrical) that promote the fusion of cell membranes. An 8:1 proportion was used in the example in Section 6.4. It is often preferred that the same species of animal serve as the source of the somatic and myeloma cells used in the fusion procedure. Fusion methods have been described by Kohler and Milstein (1975, Nature 256:495-497; 1976, Eur. J. Immunol. 6:511-519), and by Geffer et al. (1977, Somatic Cell Genet. 3:231-236). The fusion-promoting agent used by those investigators were Sendai virus and polyethylene glycol (PEG), respectively. The fusion procedure of the example of the present invention is a modification of the method of Kohler and Milstein, supra.

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#### 5.5. ISOLATION OF CLONES AND ANTIBODY DETECTION

Fusion procedures usually produce viable hybrids at very low frequency, about  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . Because of the low frequency of obtaining viable hybrids, it is essential to have a means to select fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among the other resulting fused cell hybrids is also necessary.

Generally, the fused cells are cultured in selective media, for instance HAT medium containing hypoxanthine, aminopterin and thymidine. HAT medium permits the

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proliferation of hybrid cells and prevents growth of unfused myeloma cells which normally would continue to divide indefinitely. Aminopterin blocks de novo purine and pyrimidine synthesis by inhibiting the production of tetrahydrofolate. The addition of thymidine bypasses the block in pyrimidine synthesis, while hypoxanthine is included in the media so that inhibited cells can synthesize purine using the nucleotide salvage pathway. The myeloma cells employed are mutants lacking hypoxanthine phosphoribosyl transferase (HPRT) and thus cannot utilize the salvage pathway. In the surviving hybrid, the B lymphocyte supplies genetic information for production of this enzyme. Since B lymphocytes themselves have a limited life span in culture (approximately two weeks), the only cells which can proliferate in HAT media are hybrids formed from myeloma and spleen cells.

To facilitate screening of antibody secreted by the hybrids and to prevent individual hybrids from overgrowing others, the mixture of fused myeloma and B lymphocytes is diluted in HAT medium and cultured in multiple wells of microtiter plates. In two to three weeks, when hybrid clones become visible microscopically, the supernatant fluid of the individual wells containing hybrid clones is assayed for specific antibody production. The assay must be sensitive, simple and rapid. Assay techniques include radioimmunoassays, enzyme immunoassays, cytotoxicity assays, and plaque assays.

#### 5.6. CELL PROPAGATION AND ANTIBODY PRODUCTION

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A sample of the hybridoma can be injected into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The



injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated in vitro in laboratory culture vessels. The culture medium, containing high concentrations of a single specific monoclonal antibody, can be harvested by decantation, filtration or centrifugation.

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#### 5.7. IN VITRO IMMUNOSEROLOGICAL APPLICATIONS

Generally stated, the use of the monoclonal antibodies described herein can be extended to the screening of human biological fluids for the presence of the specific antigenic determinant recognized by that monoclonal antibody. In vitro immunoserological evaluation of blood withdrawn from patients thereby permits non-invasive diagnosis of various vascular diseases. By way of illustration, blood (including plasma) can be taken from a patient and assayed for the specific antigen, such as soluble fibrin. The monoclonal antibodies may be used in radioimmunoassays or enzyme-linked immunoassays to assay for the presence of fibrin. The levels of fibrin or FDP present may be indicative of a vascular disease or may be used to monitor the progress of fibrinolytic therapy.

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It is preferred in the practice of this invention that the diagnostic assay be carried out in solution, that is, the antigen-antibody reactions take place in a aqueous medium. A preferred assay to detect and measure fibrinogen fragments, fibrin or fibrin fragments uses the monoclonal antibodies of this invention and comprises reacting a fluid sample from a human patient containing an unknown amount of fibrinogen and/or fibrin and/or fibrin fragments with a monoclonal antibody of this invention which is specific for these compounds in which the sample has been labelled with a

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radioactive or similar label. The reaction product of this reaction is then reacted with an antibody against the monoclonal antibody. This antibody may be a polyclonal anti-mouse IgG antibody. Once any antibody-monoclonal antibody-fibrinogen (or fibrin) fragment complex has been formed the complex should be separated from the other unreacted solution components. The presence of a label in the reacted and separated components is indicative of the presence of these fragments in the fluid sample of the human patient. The amount of the fragments present in the sample may be determined by comparing the amount of label present against a dose-response curve generated using known amounts of fibrinogen/fibrin fragments.

Another assay to detect and measure fibrinogen/fibrin fragments utilizes the monoclonal antibodies of this invention with a Western Blot analysis of a fluid sample, i.e. plasma, from a human patient. Generally stated, this assay comprises:

- a. electrophoretically separating blood plasma components containing an unknown amount of fibrinogen fragments from a plasma sample from a human patient;
- b. transferring said separated plasma components to nitrocellulose;
- c. treating the nitrocellulose with a labeled monoclonal antibody of the invention;
- d. removing unreacted monoclonal antibody of step c; and
- e. measuring the amount of label activity in any labeled monoclonal antibody-fibrinogen fragment reaction products present on the nitrocellulose, and quantitatively determining the amount of fibrinogen fragments present in the sample by comparing

with a standard dose-response curve generated with standards containing known amounts of said fibrinogen fragments.

Another assay using the monoclonal antibodies of this invention to detect fibrinogen/fibrin fragments comprises:

- a. providing a body fluid sample containing an unknown amount of fibrinogen fragments to a plastic container;
- b. reacting a labeled monoclonal antibody of the invention with the fluid sample of step a;
- c. removing any unreacted monoclonal antibodies of step b and;
- d. measuring the amount of label activity in the container, and quantitatively determining the amount of fibrinogen fragments present in the sample by comparing with a standard dose-response curve generated with standards containing known amounts of said fibrinogen fragments.

This assay utilizes the characteristic affinity of proteins to plastics such as PVC.

#### 5.8. IN VIVO DIAGNOSTIC AND THERAPEUTIC USES FOR MONOCLONAL ANTIBODIES TO FIBRIN AND ITS DEGRADATION PRODUCTS

##### 5.8.1. CLOT/VASCULAR DISEASE LOCALIZATION

The monoclonal antibodies of this invention are capable of targeting fibrin clots or aggregation of fibrin in vivo. They can, therefore, be used in humans for localization of possible tissue or vascular damage and for monitoring of vascular diseases. For this application, it is preferable to use purified monoclonal antibodies. Preferably, purification may be accomplished by HPLC methods. Purification of monoclonal antibodies for human

administration may also be accomplished by ammonium sulfate or sodium sulfate precipitation followed by dialysis against saline and filtration sterilization (Miller et al., 1982, In Hybridomas in Cancer Diagnosis and Therapy, (1982) supra p. 134, and by Dillman et al., Id. at p. 155, which are hereby incorporated by reference). Alternatively, immunoaffinity chromatography techniques may be used to purify the monoclonal antibodies.

The purified monoclonal antibodies can be labelled with radioactive compounds, for instance, radioactive iodine, and administered to a patient intravenously. The antibody can also be labelled with a magnetic probe. NMR can then be utilized to pinpoint the clot. After localization of the antibodies at the clot or fibrin aggregation they can be detected by emission tomographical and radionuclear scanning techniques thereby pinpointing the location of the thrombotic or fibrin encapsulated tumor. Experimental radioimmunodetection with monoclonal antibodies by external scintigraphy has been reported by Solter et al. (Id., at p. 241) hereby incorporated by reference.

By way of illustration, the purified monoclonal antibody is suspended in an appropriate carrier, e.g., saline, with or without human albumin, at an appropriate dosage and is administered to a patient. The monoclonal antibodies are preferably administered intravenously, e.g., by continuous intravenous infusion over several hours, as in Miller et al., incorporated by reference, supra.

#### 5.8.2. TREATMENT OF VASCULAR DISEASES WITH MONOCLONAL ANTIBODY CONJUGATES

The monoclonal antibodies of this invention can be used in conjunction with a broad spectrum of pharmaceutical agents such as : radioactive compounds (e.g.,  $I^{125}$ ,  $I^{131}$ ,  $^{99m}Tc$ ) various antibiotics (e.g., daunomycin, adriamycin, chlorambucil) or cardiovascular agents (e.g. beta blockers,

calcium channel blockers, t-PA, urokinase or streptokinase). For various reviews on the subject, see Bale *et al.*, 1980, Cancer Research 40:2965-297; Ghose and Blair, 1978, J. Natl. Cancer Inst. 61(3):657-676; Gregoriadis, 1977, Nature  
5 265:407-411; Gregoriadis, 1980 Pharmac. Ther. 10:103-108; Trouet *et al.*, 1980, Recent Results Cancer Res. 75:229-235.

The methods used for binding these agents to the monoclonal antibody molecule can involve either non-covalent or covalent linkages. Since non-covalent bonds are more  
10 likely to be broken before the antibody complex reaches the target site, covalent linkages are preferred. For instance, a carbodiimide bond can be formed between the carboxy groups of the pharmaceutical agent and the amino groups of the antibody molecule. Bifunctional agents such as dialdehydes  
15 or imidoesters can be used to link the amino group of a drug to amino groups of the antibody molecule. The Schiff base reaction can be used to link drugs to antibody molecules. This method involves the periodate oxidation of a drug or  
20 cytotoxic agent that contains a glycol or hydroxy group, thus forming an aldehyde which is then reacted with the antibody molecule. Attachment occurs via formation of a Schiff base with amino groups of the antibody molecule. Additionally, drugs with reactive sulfhydryl groups have been coupled to antibody molecules.

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## 6. EXAMPLES

### 6.1. FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS

Fibrinogen degradation products were prepared by incubating fibrinogen with plasmin at 37°C for time periods  
30 ranging from 10 minutes to 3 hours as described by Varadi & Patthy, Biochem 22:2440-2446 (1983). At the desired time, fibrinogenolysis was stopped by the addition of Trasylol at 100 KIU/ml and epsilon-aminocaproic acid to a final  
35 concentration of 20 mM.

Fibrin degradation products were prepared by the addition of thrombin (4 NIH units per ml) to a fibrinogen solution (5 mg/ml in Tris saline buffer, pH 7.4) containing 10 mM  $\text{CaCl}_2$ , 0.25 mg/ml plasminogen, and 50 plouge units/ml urokinase. The mixture was incubated at 37°C and the plasmin digestion terminated at different time intervals.

Fibrinogen chains alpha, beta and gamma were prepared by incubation of fibrinogen with mercaptoethanol (5% final concentration) at 90°C for 5 minutes.

Purified fibrinogen fragments E and D were a gift from Dr. Frank Castellino although they may also be produced by the method disclosed by Pizzo, S., et al., 1973, J. Biol. Chem. 248, 4584.

#### 6.2. OTHER REAGENTS

All tissue culture reagents were purchased from M.A. Bioproducts, with the exception of RPMI 1640 medium, fetal bovine serum and horse serum which were obtained from Gibco. Bovine serum albumin and plasminogen-free bovine thrombin were both obtained from Miles Laboratories. Human urokinase was obtained from Calbiochem. All other reagents were of analytical grade.

#### 6.3. IMMUNIZATION SCHEDULE

Balb/c mice were immunized, intraperitoneally, with human fibrin (50 ug) in Freund's complete adjuvant. A booster injection was given, intraperitoneally (i.p.), after 4 weeks, using the same amount of antigen in Freund's incomplete adjuvant. A final booster injection was administered intravenously, 4 weeks later and 3 days before the fusion. The serum titer levels against the antigen were measured 24 hours before the fusion and only those mice which gave positive assay results at serum dilutions of greater than  $10^5$  were used.

#### 6.4. FUSION TO PRODUCE HYBRIDS

Spleen cells from the immunized mice were fused, at a ratio of 8:1, with cells from an 8-azaquinine-resistant NS-1 myeloma cell line. The fused cells were cultured in a selection media, containing Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum (gamma-globulin free)/10% horse serum (gamma-globulin free)/10% (v/v) NCTC 109/2 mM glutamine/1 mM sodium pyruvate/ $10^{-4}$  M hypoxanthine/ $1.6 \times 10^{-5}$  M thymidine/1 mM sodium oxaloacetate/0.511 mM pyruvic acid/0.2 unit per ml bovine insulin (HY media)/ $8 \times 10^{-7}$  M aminopterin (HAT media).

After 2-3 weeks, those wells with visible growth were assayed for antibody production using an ELISA method.

Antibody producing cell lines were recloned three times by limiting dilution. Monoclonal antibody producing cell lines were obtained from wells diluted to 1 cell/well during the tertiary cloning phase.

More specifically, it was only the spleens of those BALB/c mice which showed a positive immune response against the human fibrin antigen which were used in the fusion with the NS-1 myeloma cells. In the primary cloning phase, 121 wells showed vigorous growth; of these, 18 were shown by ELISA to be producing antibody to the antigen. The 18 hybridomas were cloned three times by limiting dilution and eventually 3 stable antibody-producing cell lines were isolated after the tertiary cloning stage. These cell lines were designated DG1, DG2 and DG3.

#### 6.5. PURIFICATION OF MONOCLONAL ANTIBODIES

Tissue culture media from the antibody producing cell line was applied to a Sepharose-Protein A affinity column at a flow rate of 0.5 ml per minute. The column was washed with phosphate buffered saline, pH 7.2, until the eluent absorbance at a 280 nm wavelength reached a steady baseline near zero. The antibody was eluted from the column with a

0.5 M glycine solution, pH 3. All eluted fractions were adjusted to pH 7 with 1.0 M Tris, pH 7.5. Antibody containing solutions were pooled, dialysed against phosphate buffered saline, pH 7.2, and stored at 4°C in the presence of 0.005% Merthiolate (Sigma). Monoclonal antibody was also obtained from the ascites fluid of mice which had been injected with  $10^5$  of the antibody producing hybridoma cells. Antibody from ascites was purified by ion-exchange HPLC (Gemski M. J., et al., 1985, Single step purification of monoclonal antibody from murine ascites and tissue culture fluids by an ion exchange high performance liquid chromatography. Biotechniques 3(5): 378-384). Homogeneity of the purified antibodies was verified by gel electrophoresis and by HPLC.

15

#### 6.6. IMMUNOSPECIFICITY DETERMINATION

The immunoreactivity of the monoclonal antibodies to fibrin, fibrinogen and their respective degradation products was first assessed using the ELISA technique. An immunoblotting technique was also employed in which samples of fibrinogen and fibrin degradation products were electrophoresed on 5% polyacrylamide gels. Protein transfer from the acrylamide gels to nitrocellulose sheets was performed in an electroblotting cell (Bio-Rad) with a transfer medium of 20 mM Tris, 120 mM glycine, pH 8.0, containing 20% methanol. Electroblothing was allowed to proceed for 48 hours at 4°C. The immunoblots were blocked with a 2% BSA solution in TBS, pH 7.4, at 37°C for 90 minutes. The nitrocellulose sheets were then rinsed with TBS and incubated for 90 minutes with monoclonal antibody (cell supernatant) diluted 1:1 (v/v) with 2% BSA in TBS. The nitrocellulose sheets were then treated with a 1000-fold dilution of peroxidase-conjugated rabbit anti-mouse IgG in 2%



BSA in TBS. After rinsing, the antigen/antibody complex was visualized after incubating with 0.025% hydrogen peroxide and 4-chloro-1-naphthol.

As indicated by Western Blotting analysis, the three antibodies, DG1, DG2, and DG3, crossreacted with the soluble fibrin. Only DG1 did not crossreact with native fibrinogen, but each of the antibodies bound to one of a number of the fibrin/fibrinogen degradation products. Confirmation of the specificity for a particular degradation product was obtained by repetition of the blotting experiment using purified samples of the fragment. The results are detailed in the following Table I.

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TABLE I

Immunospecificity of the Monoclonal Antibodies DG1 and DG2,  
5 to Fibrin, Fibrinogen and their Plasmin Degradation Product.

	Antigen	Immunoreactivity <sup>a</sup>	
		DG1	DG2
10	Fibrin	+++	+++
	D-dimer	+++	+
	Fibrinogen	-	+++
	Fragment E	-	+
	Fragment D	+	++
15	Fragment X	+	+++
	Fragment Y	+	+++
	A chain	+	+++
	B chain	-	-
	Gamma chain	-	-

20 a The immunoreactivities were determined by an immunoblotting experiment as described in the experimental section. The presence of an antibody-antigen complex was determined using a peroxidase-conjugated antimouse antibody and a colorimetric chloronaphthol/hydrogen  
25 peroxide reagent. The results are expressed in terms of the intensity of the colored complex formed: +++ maximum crossreactivity; + minimal crossreactivity; ++ crossreactivity; - no crossreactivity.

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#### 6.7. ANTIBODY SUBCLASSING

Antibody subclassing was performed using the Enzyme-Linked Immunoabsorbant Assay (ELISA) method. Rabbit anti-mouse IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub> and IgG<sub>3</sub>, in addition to rabbit  
5 antimouse kappa and lambda light chains, were obtained for this assay from Zymed Laboratories, San Francisco.

To each well of a 96 well PVC microtiter plate (COSTAR) was added 5 ug of the antigen to be tested in 200 ul of a borate/saline buffer, pH 8.3. The plates were incubated  
10 at room temperature for 3 hours. After washing several times with phosphate buffered saline, 200 ul of a 1% bovine serum albumin solution was added to each well and the plate incubated at 37°C for 1 hour. Antibody-containing solutions (200 ul) were then added to the wells and incubated for 90  
15 minutes at 37°C. The wells were thoroughly washed with PBS. Alkaline phosphatase conjugated to Rabbit anti-mouse IgG in PBS/1% BSA, pH 8.0 was added and the plates incubated for a further 90 minutes at 37°C. Enzyme substrate, p-nitrophenyl phosphate (0.4 mg/ml), dissolved in 10% diethanolamine/0.83  
20 mM MgCl<sub>2</sub>, pH 9.8, was then added to each well. After 30 minutes, the reaction was quenched by addition of 50 ul of 3 M NaOH. The absorbance of each well was determined at a wavelength of 405 nm using an Artek automatic plate reader.

Subtyping by ELISA, using standard antimouse IgG<sub>1</sub>,  
25 IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub>, showed that all three antibodies, DG1, DG2 and DG3, belonged to the IgG<sub>1</sub> class. Likewise all three antibodies gave positive ELISA results with anti-mouse kappa light chains and were negative with anti-mouse lambda light chains.

30 HPLC chromatograms, obtained on calibrated Protein-Pak columns, showed just one peak at 150,000 daltons.

### 6.8. BINDING STUDIES IN VITRO

Binding studies of the purified monoclonal antibodies were performed by solid-phase radioimmunoassay. Antibodies were labelled using Iodobeads (Pierce Chemicals, Rockford) and Na<sup>125</sup>I according to the manufacturers' instructions. The radiolabelled antibodies were separated from excess <sup>125</sup>I by passage over a Sephadex G-150 column followed by extensive dialysis against phosphate buffered saline (PBS).

The avidity of the <sup>125</sup>I-labelled antibodies for the various fibrin/fibrinogen fragments were measured using an adaptation of the method described by Frankel and Gerhard (1979, Mol. Immunol. 16:101-106). The protein or fragment of interest was coated onto the wells of a microtiter plate at a concentration of 10 ug/ml in Borate/Saline, pH 8.0. The wells were then washed and postcoated with 1% BSA in PBS.

Various concentrations of the <sup>125</sup>I-labelled antibody in 1% BSA were added to the coated wells and allowed to incubate at 37°C for 90 minutes. The antibody solution was removed and wells washed extensively with PBS/Tween 20. The wells were then cut from the plate and placed in suitable vials for counting in a Beckman model 5500 gamma counter. For each antibody concentration, triplicate experiments were performed and the results averaged. Controls were performed in which the same concentration of <sup>125</sup>I-labelled antibody was incubated in the wells of a plate coated only with BSA.

The level of radioactivity found in each well was converted to the concentration of antibody bound. Binding constants were calculated from the following equation:

$$\frac{[A]_B}{[A]_F} = sK_a [P]_T - nK_a [A]_B$$

where  $[A]_B$  is the concentration of antibody bound to the plate and  $[A]_F$  is the free concentration of antibody. The parameters  $s$ ,  $K_a$ ,  $[P]_T$ , and  $n$  represent the valence of the

antigen, the association constant of the ligand to the antibody, the total antigen concentration, and the valence of the antibody, respectively. A plot of  $[A]_B/[A]_F$  vs  $[A]_B$  yields a slope of  $-2Ka$ .

5

#### 6.9. DETERMINATION OF BINDING CONSTANTS

Prior to radioimmunoassay (RIA) for the determination of the avidity constants, the effect of iodination on the binding efficiency of the antibody was tested using the standard ELISA technique. A decreased binding efficiency of approximately 10% was determined. A modified Scatchard plot of the binding data for DG1 to fibrin and fibrinogen is shown in Figure 1. Dissociation constants ( $K_D$ ) was determined from the slope of the lines and calculated to be  $1.20 \times 10^{-9}$  M and  $3.20 \times 10^{-8}$  M for fibrin and fibrinogen, respectively. Similar experiments were performed to determine the binding constants of DG2 to fibrinogen and fibrin. The results are listed in the following Table II.

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TABLE II

The Binding Constants,  $K_D$ , for Antibodies, DG1 and DG2 for  
 5 Various Antigens<sup>a</sup>

Antigen	$K_D$ ( $\times 10^{-8}$ M)	
	DG1	DG2
10 Fibrin <sup>b</sup>	0.12	1.07
Fibrinogen	3.20	0.70
Soluble Fibrin <sup>c</sup>	3.00	0.65

15 <sup>a</sup>  $K_D$  values were determined using a solid phase RIA and a modified form of the Scatchard analysis method.

<sup>b</sup> Fibrin coated wells were prepared by thrombin treatment of fibrinogen-coated wells, as described in methods section.

<sup>c</sup> Soluble fibrin refers to that portion of a fibrin clot  
 20 solubilised by 4 M urea.

The affinity of antibody DG1 for fibrinogen is approximately 30 fold less than it is for fibrin, and it remains to be  
 25 determined if the affinity for fibrinogen is due to an alteration of conformation induced by the binding of fibrinogen to the plastic surface. The antibody DG2 is clearly directed to an epitope on the native fibrinogen and the decreased but sustained affinity observed for fibrin is  
 30 probably due to a modification of the epitope induced by fibrin formation.

6.9.1. DETERMINATION OF THE FIBRIN SPECIFICITY  
USING A COMPETITIVE ASSAY

A competitive assay system was developed to further  
5 investigate the crossreactivity of DG1 with fibrinogen in  
solution, as opposed to fibrinogen bound to a plastic  
surface. Solutions containing various levels of fibrinogen  
were added to fibrin coated wells followed by a solution  
containing constant levels of  $^{125}\text{I}$ -labelled antibody.  
10 Following an incubation period of 90 minutes at 37°C, the  
solutions were removed from the wells and after extensive  
washing the bound activity was measured in a gamma counter.  
A control experiment was performed in which the antibody, DG1  
was replaced by DG2. In both experiments subsaturating  
15 levels of antibody, as determined in a preliminary  
experiment, were used. In the case of DG1, fibrinogen levels  
as high as 5 mg/ml did not cause any significant decrease in  
the amount of antibody binding to fibrin (Fig 2). The level  
of fibrin bound to the individual wells of the assay plate  
20 was determined using an  $^{125}\text{I}$ -labelled fibrinogen tracer, to  
be 500 ng. Therefore, in this particular competition assay  
the fibrinogen level of 5 mg/ml (0.5 mg/well) represents a  
1000 fold excess over the fibrin antigen and indicates fibrin  
specificity of the monoclonal antibody DG1.

25 This observation of fibrin specificity also indicates  
that a conformational change occurs when fibrinogen binds to  
the plastic surface and partially exposes the neoantigenic  
site to which DG1 is directed. Figure 2 also shows that the  
antibody DG2 is clearly specific for an epitope on the native  
fibrinogen molecule. Approximately 50% of the antibody bound  
30 to fibrinogen after the addition of 500 ng of the antigen to  
the fibrin coated well, and the antibody was almost  
completely removed in the presence of excess amounts of  
fibrinogen (Fig 2).

An additional competition assay was performed to test the fibrin specificity of the antibody DG1. In this assay, fibrinogen solutions were added to tubes containing a suspension of fibrin, formed by sonication of a crosslinked  
5 fibrin clot. The level of  $^{125}\text{I}$ -labelled antibody which binds to the fibrin was determined after extensive washing and centrifugation of the fibrin clots. Figure 3 shows that increasing fibrinogen levels does not inhibit binding of DG1 to the fibrin clot, whereas the same fibrinogen  
10 concentrations cause a significant decrease in the levels of DG2 which bind to fibrin.

6.10. THE EFFECT OF FIBRIN BOUND MONOCLONAL ANTIBODIES  
DG1 and DG2 ON THE ACTIVITY OF TISSUE TYPE  
PLASMINOGEN ACTIVATOR (t-PA)

15 The effect of fibrin bound antibodies, DG1 and DG2, on t-PA activity was determined using an adaptation of the spectrophotometric assay described by Drapier et al., (1979, Regulation of plasminogen activator secretion in mouse peritoneal macrophages. Biochimie 61:463-471). The t-PA  
20 mediated generation of plasmin was followed by measuring the change in absorbance of the test solution as p--nitroaniline (p-NA) was released from the chromogenic substrate, S-2251, by the amidolytic action of plasmin. The assay was performed after preincubation of the fibrin coated wells with levels of  
25 antibody ranging from zero to concentrations well in excess of saturating levels (Fig. 4). The kinetics of the reaction were evaluated by determining the rate of release of p-NA, calculated from the slopes of the respective lines. The  
data, as represented in Fig. 5, show a steady decline in t-PA  
30 activity with increasing levels of fibrin bound antibody, DG1. No additional inhibition of the t-PA activity could be induced by increasing antibody levels in excess of those shown. As Fig. 4B and the kinetic data summarized in Fig. 5 show, the antibody DG2 has no effect on t-PA activity. Due



to the relatively low affinity of this antibody to fibrin, the experiment was repeated at antibody levels up to 100 times those used with DG1 and even at these elevated levels no effect was observed.

5        In another series of experiments using an  $^{125}\text{I}$ -fibrin plate assay, the effect of the antibodies DG1 and DG2 on the fibrinolytic activity of t-PA was determined. The fibrin coated wells were preincubated with solutions containing saturating levels of antibody. After removal of unbound  
10 antibody the fibrinolytic activity was measured. In control experiments, the amount of bound activity was determined by trypsin digestion of the fibrin film. In those wells preincubated with antibody DG1, only 60% of the bound activity was released after a 3 hour incubation at 37°C with  
15 t-PA and plasminogen (see Table III). Wells not preincubated with antibody released 90% of the bound activity over the same period. Wells, preincubated with antibody DG2, did not show any fibrinolytic inhibition. The t-PA activity measured was almost identical to that measured in the absence of  
20 antibody (see Table III).

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TABLE III

 $^{125}\text{I}$ -Fibrin Plate Competition Assay.

5 The Effect of Monoclonal Antibodies DG1 and DG2 on Fibrinolysis.

	<u>Antibody</u>	<u>Activity (cpm)<sup>a</sup></u>
	DG1	70,810
10	DG2	100,900
	No antibody	120,904

15 <sup>a</sup> Values represent the  $^{125}\text{I}$  activity released after 1 hour of incubation with t-PA and plasminogen.

6.10.1. PREPARATION OF PLASMINOGEN

20 Plasminogen was purified from human plasma by affinity chromatography on Sepharose 4B-L-lysine (Pharmacia) using a modification of the Deutsch and Mertz method (1970, Plasminogen: Purification from human plasma by affinity chromatography. Science 170: 1095-1096) as described by Brockway and Castellino (1972, Measurement of the binding of 25 antifibrinolytic amino acids to various plasminogens. Arch Biochem Biophys 151: 194-199). Tissue plasminogen activator (t-PA) was isolated from the tissue culture fluid of a Bowes' melanoma cell line, generously provided by Dr. D. Rifken (Rockefeller University). The activator was purified using a 30 modification of the method described by Collen et al. (1981, Purification and characterisation of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 256: 7035). Briefly, tissue culture fluid from the 35 cell line was chromatographed on two affinity columns,

Sepharose-benzamidine followed by Sepharose Concanavalin A. Final purification of the activator was performed by gel filtration on a Sephacryl-S-200 column.

5        6.10.2. SPECTROPHOTOMETRIC ASSAY FOR t-PA ACTIVITY

The rate of activation of plasminogen to plasmin by t-PA was measured by a chromogenic assay using the plasmin specific substrate, S-2251. The assay was performed using a modification of the method described by Drapier et al.,  
10 supra. A TBS solution (pH 7.4) containing S-2251 (0.5 mM), plasminogen (1.0 mg/ml), BSA (2.5 mg/ml) and t-PA (2.0 Ploug units/ml) was added to fibrin coated wells of a microtiter plate. The plates were incubated at 37°C and the rate of plasmin formation determined by measuring the change in  
15 absorbance of the solutions at 405 nm, as described by Drapier, supra.

6.10.3. <sup>125</sup>I-FIBRIN PLATE ASSAY

The fibrinolytic activity of t-PA was measured using a  
20 modified version of the <sup>125</sup>I-fibrin plate assay method, described by Unkeless et al. (1973, An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. J. Exp. Med. 137: 85-111). In brief, <sup>125</sup>I-labelled  
25 fibrinogen (1300 cpm/ng) was diluted with unlabelled fibrinogen so that approximately  $6.5 \times 10^5$  cpm was added to each well of a 24-well tissue culture plate (Falcon). The  
fibrinogen coated on each well was converted to fibrin by incubation with 1 ml of a thrombin solution (2 NIH units/ml) for 1 hour at 37°C. The plates were allowed to dry at 40°C  
30 for 48 hours. Plasminogen (5 ug/ml) was added to a solution containing tPA (2 ploug units/ml) and 1ml aliquots of this solution were incubated in the fibrin coated wells at 37°C. Samples (0.05 ml) were removed at 30 minute intervals and the activity rel as d was measur d in a gamma c unter (Beckman  
35 Model 5500).

6.10.4. A MODEL ANIMAL SYSTEM

In vivo monitoring of fibrin formation and the effect of monoclonal antibodies DG1 and DG2 on fibrinolysis induced by tissue plasminogen activators, were examined in a model animal system.

Fibrinogen, radiolabelled with  $^{125}\text{I}$ , (approximately 21 uCi in 300 ul of saline) was injected via the saphenous vein into an anaesthetised rat and allowed to circulate for 30 minutes. A lead template was placed over the chest area, and a probe connected to a gamma counter was inserted into the template to monitor the  $^{125}\text{I}$  activity. Fibrin clot formation was induced by intravenous injection of thrombin (700 NIH units/Kg/h) in saline (5 ml). Plasmin dissolution of the clot was inhibited by subcutaneous injection of epsilon-aminocaproic acid (200 mg) 15 minutes prior to thrombin infusion.  $^{125}\text{I}$ -labelled monoclonal antibodies were injected intravenously when the count rate indicated that clot formation was complete. During each experiment 1-minute counts were recorded every 5 minutes and blood samples were removed via the carotid artery at suitable intervals.

Using this system, it was demonstrated that clot formation and subsequent dissolution could be monitored successfully using radio-labelled fibrinogen. Figure 6 shows the change in activity in the lungs when thrombin is injected. 30 minutes after  $^{125}\text{I}$ -labelled fibrinogen is administered. When clot formation is complete (approximately 30 minutes after thrombin infusion), the activity begins to decrease as the clot dissolves and reaches a baseline value after 60 minutes. Inhibition of this plasmin degradation can be induced by introduction of epsilon-aminocaproic acid (200 mg) prior to thrombin injection.

Significantly, when monoclonal antibody (DG1) is injected at time  $t=15$ , a change in the pattern of activity is observed (see Figure 6). The

pattern, deviated in that the onset of the clot dissolution was delayed by inhibition by the bound antibody.

To demonstrate fibrin specificity of the antibody, another experiment was performed.

5       a) Monoclonal antibody (DG1), radiolabelled with  $^{125}\text{I}$  was injected at  $t=0$  and allowed to circulate for 2h; 1 minute counts were taken every 5 minutes and the results plotted as before (Figure 6). The activity settled at approximately 20,000 CPM. No thrombin was added in this experiment and no  
10 accumulation of activity was observed. The purpose of this experiment was to determine the baseline activity when a certain dose of labelled antibody was administered.

      b) In the subsequent experiment, clot formation was induced and maintained as before by administration of  
15 thrombin and EACA respectively to an animal with circulating  $^{125}\text{I}$  labelled fibrinogen. The same quantity of labelled DG1, as used in the previous experiment, was administered 40 minutes after clot formation. An increase in activity of 30,000 CPM was observed which remained almost constant for a  
20 further 40 minutes until termination of the experiment. (Figure 4) (Section 6.9). If the antibody had not been fibrin specific there would have been an expected increase in activity of approximately 20,000, however, the increase was 50% in excess.

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#### 7. DEPOSIT OF HYBRIDOMAS

The following hybridomas have been deposited with the American Type Culture Collection, Rockville, MD, and have been assigned the listed accession numbers:

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<u>Hybridoma</u>	<u>Accession Number</u>
DG1	HB9186
DG2	HB9187
DG3	HB9188

5           The present invention is not to be limited in scope by  
the hybridomas deposited since each deposited embodiment is  
intended as a single illustration of one aspect of the  
invention and any cell line which is functionally equivalent  
10 is within the scope of this invention. Indeed, various  
modifications of the invention in addition to those shown and  
described herein will become apparent to those skilled in the  
art from the foregoing description and accompanying drawings.  
Such modifications are intended to fall within the scope of  
15 the appended claims.

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We claim:

1. A monoclonal antibody against fibrin, produced by  
5 a hybridoma formed by a fusion of a myeloma cell and a cell  
capable of producing an antibody against fibrin.

2. A monoclonal antibody against fibrinogen, produced  
by a hybridoma formed by a fusion of a myeloma cell and a  
10 cell capable of producing an antibody against fibrinogen.

3. A monoclonal antibody against fibrin D dimer,  
produced by a hybridoma formed by a fusion of a myeloma cell  
and a cell capable of producing an antibody against fibrin.

15 4. A monoclonal antibody against fibrinogen  
proteolytic fragments, produced by a hybridoma formed by a  
fusion of a myeloma cell and a cell capable of producing an  
antibody against fibrin.

20 5. The monoclonal antibody of claims 1, 2, 3 or 4  
wherein said cells capable of producing antibodies are  
obtained from a mouse.

25 6. The monoclonal antibody of claims 1, 2, 3 or 4  
wherein said myeloma cell is a mouse myeloma cell.

7. The monoclonal antibody of claims 5 wherein the  
cell capable of producing antibody is selected from the group  
consisting of spleen cells, lymph node cells, and peripheral  
30 blood lymphocytes.

8. The monoclonal antibody of claim 1 wherein said  
monoclonal antibody is capable of differentiating fibrin from  
35 fibrinogen.

9. The monoclonal antibody of claim 7 wherein said cell capable of producing antibody is derived from a BALB/C mouse spleen.

5        10. The monoclonal antibody of claim 6 wherein said myeloma cell is NS-1 myeloma cell.

10       11. A continuous cell line which produces monoclonal antibodies against a fibrinogen proteolytic fragment which fibrinogen proteolytic fragment is generated during fibrinolysis, comprising a hybridoma formed by fusing a cell capable of producing antibodies against said fibrinogen proteolytic fragment with a myeloma cell.

15       12. The continuous cell line of claim 11 wherein the fibrinogen fragment is one selected from the group consisting of fibrin, fibrin D dimer, fibrin fragment D monomer, fibrinogen fragment E, fibrinogen fragment X, and fibrinogen fragment Y.

20       13. Hybridoma cell line DG1, ATCC accession number HB9186, formed by fusing NS-1 murine myeloma cell line with a murine spleen cell obtained from a BALB/C mouse immunized with fibrin, which hybridoma cell line produces monoclonal  
25       antibodies to fibrinogen and fibrin fragments.

14. The Hybridoma cell line of claim 13 wherein the fibrin fragment is fibrin fragment D dimer.

30       15. Hybridoma cell line DG2, ATCC accession number HB9187, formed by fusing NS-1 murine myeloma cell line with a murine spleen cell obtained from a BALB/C mouse immunized with fibrin, which hybridoma cell line produces monoclonal antibodies to fibrinogen fragments.



16. The Hybridoma cell line of claim 15 wherein the fibrinogen fragment is one selected from the group consisting of fragment E, fragment D, fragment X and fragment Y.

5 17. Hybridoma cell line DG3, ATCC accession number HB9188, formed by fusing NS-1 murine myeloma cell line with a murine spleen cell obtained from a BALB/C mouse immunized with fibrin, which Hybridoma cell line produces monoclonal antibodies to a fibrinogen fragment.

10 18. The Hybridoma cell line of claim 17 wherein the fibrinogen fragment is one selected from the group consisting of fragment X and fragment Y.

15 19. The monoclonal antibody of claim 1, conjugated to an imaging compound.

20. The monoclonal antibody of claim 19 wherein the imaging compound is a radioactive compound.

20 21. A method for localizing human fibrin in vivo, which comprises:

- (a) administering the monoclonal antibody of claim 19 to a human patient with a suitable carrier;
- 25 (b) detecting localization of the monoclonal antibody in said human patient by external scintigraphy, emission tomography, radionuclear scanning or Nuclear magnetic resonance.

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22. The method according to claim 21 wherein the fibrin is present in vivo in a blood clot.

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23. A method for detecting vascular disease, which comprises: contacting the monoclonal antibody of claims 1, 2, 3 or 4 with a human fluid sample, detecting the interaction of said antibody with any antigenically-  
5 corresponding fibrinogen proteolytic fragment thereof in said sample and comparing the level of fibrinogen proteolytic fragment with the amount present in a human without vascular disease having normal levels of said fragment present.

10 24. The method according to claim 23, wherein the vascular disease is thrombosis or disseminated intravascular coagulation.

25. The method according to claim 23, wherein said  
15 fluid sample is blood.

26. The method according to claim 23, wherein said fluid sample is blood plasma.

20 27. The monoclonal antibody of claim 1, conjugated to a drug active against vascular disease.

28. A method for treating a vascular disease comprising providing an effective amount of the monoclonal  
25 antibody of claim 27 to a patient having a vascular disease.

29. The method according to claim 28 wherein the vascular disease is thrombosis or disseminated intravascular coagulation.

30 30. The method according to claim 28 wherein the monoclonal antibody together with a suitable carrier is administered to a patient.

31. An assay for detecting fibrinogen fragments in a human patient comprising:

- a. reacting a body fluid sample from said human patient which contains an unknown amount of labeled fibrinogen fragments with the monoclonal antibody of claims 1, 2, 3 or 4;
- b. reacting the reaction product of step a with labeled antibodies against said monoclonal antibody of claims 1, 2, 3 or 4;
- c. separating any reacted antibody - monoclonal antibody-fibrinogen fragment complexes from the reaction mixture of step b and,
- d. measuring the amount of label activity in any complexes separated in step c, and quantitatively determining the amount of fibrinogen fragments present in said sample by comparing with a standard dose-response curve generated with standards containing known amounts of said fibrinogen fragments.

32. The assay of claim 31 wherein the reactions of steps a and b take place in an aqueous solution.

33. The assay of claim 31 wherein the antibodies against said monoclonal antibody are anti-mouse IgG antibodies.

34. The assay of claim 33 wherein the anti-mouse IgG antibodies are polyclonal.

35. The assay of claim 31 wherein the method of separation in step c is by sepharose protein A column chromatography.

36. an assay for detecting fibrinogen fragments in a human patient comprising:

- 5 a. electrophoretically separating blood plasma components containing an unknown amount of fibrinogen fragments from a plasma sample from said human patient;
- b. transferring said separated plasma components to nitrocellulose;
- 10 c. treating said nitrocellulose with a labeled monoclonal antibody of claim 1, 2, 3 or 4;
- d. removing unreacted monoclonal antibody of step c; and
- 15 e. measuring the amount of label activity in any labeled monoclonal antibody-fibrinogen fragment reaction products present on said nitrocellulose, and quantitatively determining the amount of fibrinogen fragments present in said sample by comparing with a standard dose-response curve generated with standards containing  
20 known amounts of said fibrinogen fragments.

37. An assay for detecting fibrinogen fragments in a human patient comprising:

- 25 a. providing a body fluid sample containing an unknown amount of fibrinogen fragments to a plastic container;
- b. reacting a monoclonal antibody of claims 1, 2, 3 or 4 with the fluid sample of step a;
- 30 c. removing any unreacted monoclonal antibodies of step b; and
- d. measuring the amount of label activity in any container, and quantitatively determining the amount of fibrinogen fragments present in said sample by  
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comparing with a standard dose-response curve generated with standards containing known amounts of said fibrinogen fragments.

5        38. The assays of claims 31, 36 and 37 wherein the fibrinogen fragments include fibrin.

39. The assay of claims 31, 36 and 37 wherein the fibrinogen fragments include fibrin fragments.

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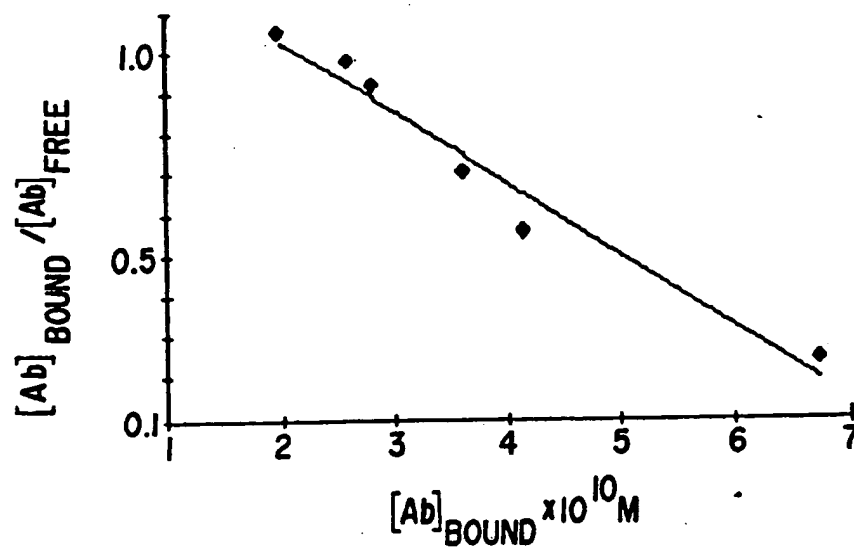


FIG. 1A

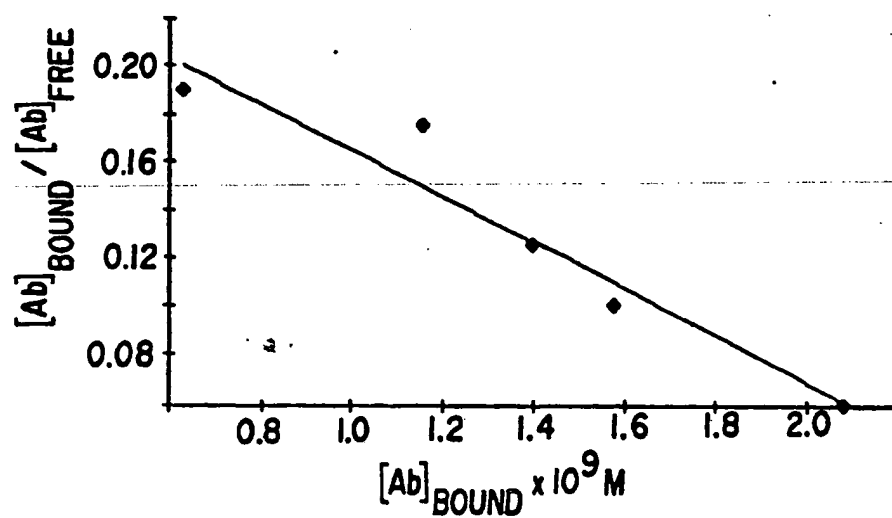


FIG. 1B

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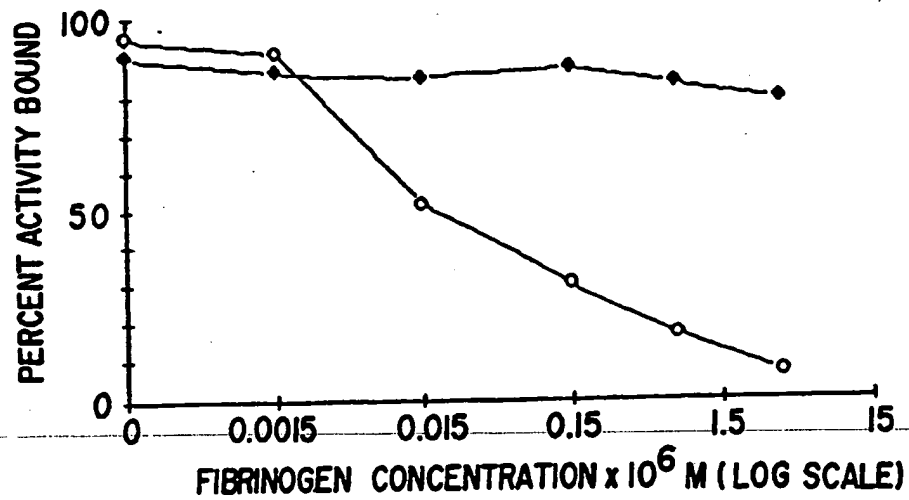


FIG. 2

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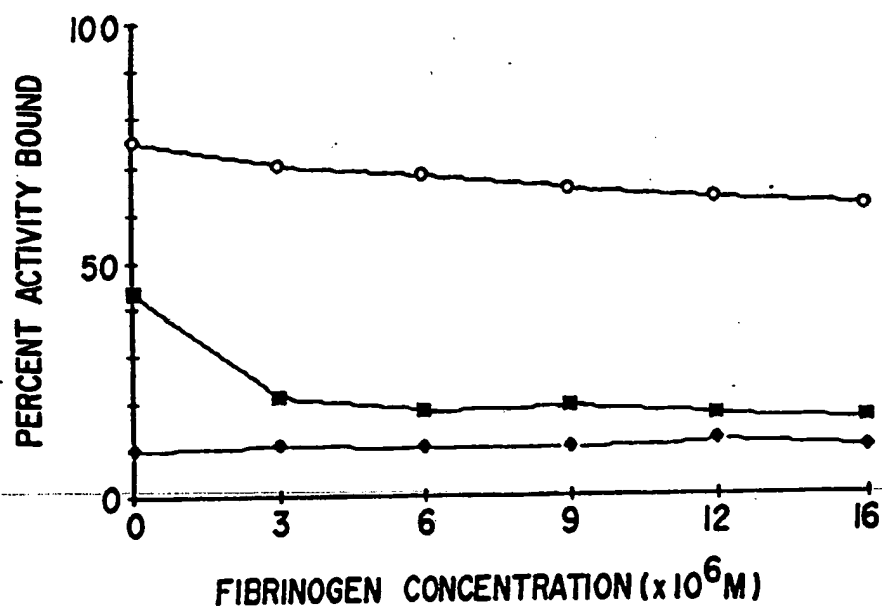


FIG. 3

SUBSTITUTE SHEET



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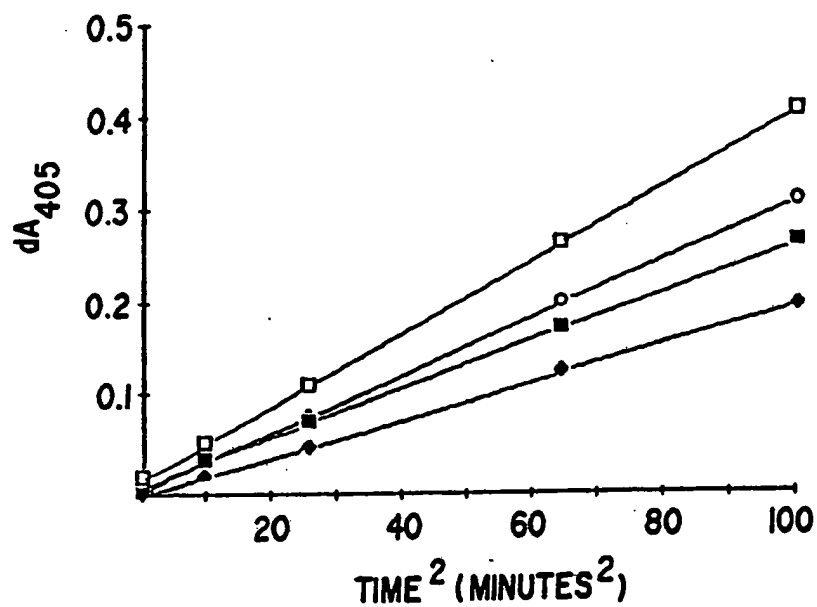
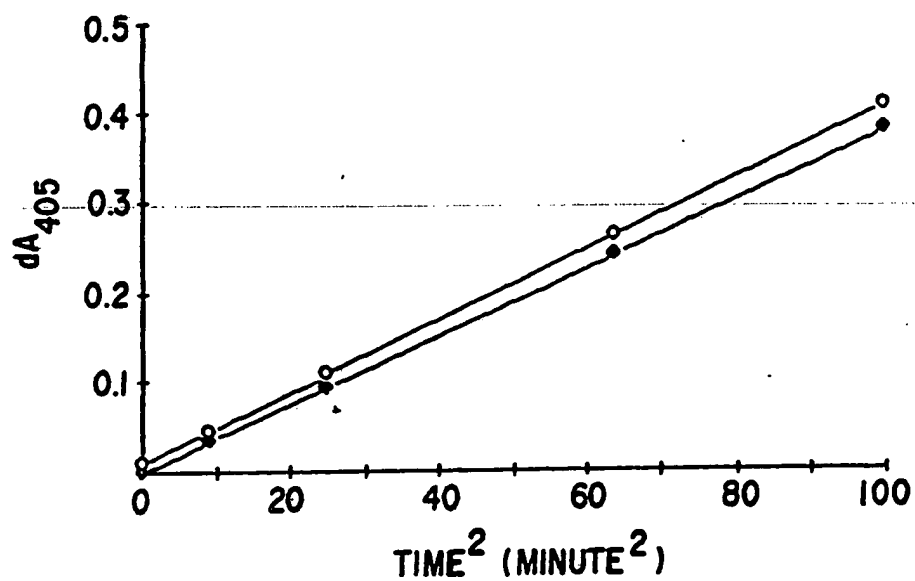


FIG. 4A

FIG. 4B  
SUBSTITUTE SHEET

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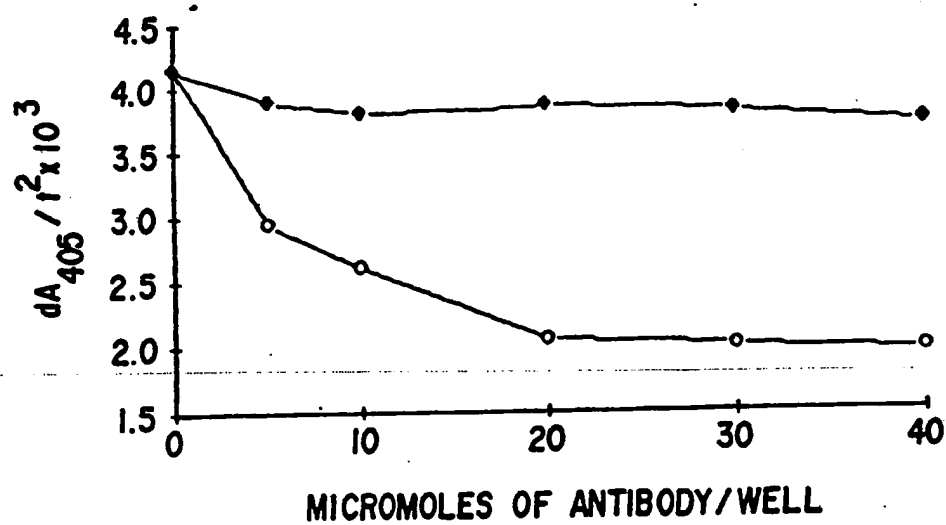


FIG. 5

SUBSTITUTE SHEET

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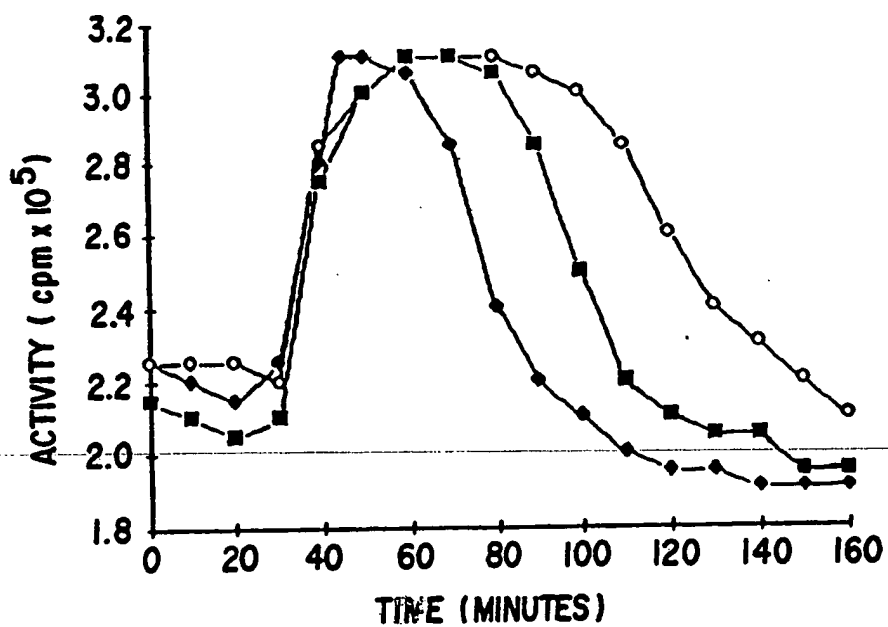


FIG. 6

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US87/02125**

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) <sup>3</sup>  
 According to International Patent Classification (IPC) or to both National Classification and IPC  
**IPC(4): A61K 39/395, 49/00; C12N 5/00; G01N 33/577**  
**U.S. Cl.: 530/387; 435/240; 436/548; 424/9, 85**

**II. FIELDS SEARCHED**

Classification System <sup>1</sup>	Minimum Documentation Searched <sup>4</sup>
U.S.	435/7, 13, 68, 172.2, 310, 948, 240.27, 424/1.1, 9, 85 535/548 530/387, 808, 809

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>6</sup>

**Computer Search:** Chemical Abstracts 1967-1987, Biosis  
1977-1987

<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup></b>		<b>Relevant to Claim No. <sup>15</sup></b>
Category <sup>11</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	
<b>X</b> <b>Y</b>	Thrombosis Research, Volume 31, 767-778, issued 1983 (Elmsford, New York, USA), D.B. Rylatt et al, "An Immunoassay For Human D Dimer Using Monoclonal Antibodies", see page 768, lines 10-16, and page 768, last paragraph.	2-7, 9-16, 23-26 31-37, 39
<b>X</b> <b>Y</b>	Science, Volume 222, 1129-1131, issued 09 December 1983 (Washington, D.C., USA), K.Y. Hui, "Monoclonal Antibodies To A Synthetic Fibrin-Like Peptide Bind To Human Fibrin But Not Fibrinogen", see page 1130, columns 2 and 3 and page 1131, column 2.	1, 5-9, 19, 20 10, 21-39
<b>X</b> <b>Y</b>	Science, Volume 229, 765-767, issued 23 August 1985 (Washington, D.C., USA) C. Bode et al, "Antibody-Directed Urokinase: A Specific Fibrinolytic Agent", see page 765, column 3, lines 1-14 and page 767, column 1.	1, 5-9 10, 23-39

\* Special categories of cited documents: <sup>13</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

**IV. CERTIFICATE**

Date of the Actual Completion of the International Search <sup>1</sup>

**22 OCTOBER 1987**

International Searching Authority <sup>1</sup>

**ISA/US**

Date of Mailing of this International Search Report <sup>1</sup>

**25 NOV 1987**

Signature of Authorized Officer <sup>10</sup>

*Esther M. Kepplinger*  
**Esther M. Kepplinger**

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages 1:	Relevant to Claim No 12
X Y	Annals of The New York Academy of Sciences, volume 408, issued 1983 (New York, N.Y., USA), J. Soria et al, "Monoclonal Antibodies That React Preferentially With Fibrinogen Degradation Products or With Cross-Linked Fibrin Split Products", see page 665, 6-13.	2,4-7, 9-13,15, 16 <u>23-29</u>
X Y	Thrombosis Research, Volume 29, 643-653, issued 1983 (Elmsford, New York, USA), B. Sola et al, "Isolation and Characterization Of A Monoclonal Antibody Specific For Fibrinogen and Fibrin of Human Origin", see page 644, lines 10-13, page 645, last paragraph and page 650, last paragraph.	2-7,9,11 12 <u>10,23-29</u>
X Y	Molecular Immunology, Volume 21, Number 1, 89-94, issued 1984 (Oxford, England), B. Kudryk et al, "Specificity of A Monoclonal Antibody For The NH <sub>2</sub> -Terminal Region Of Fibrin", see page 89, column 2 and page 94, column 1.	1,5-9,11, 12 <u>10,23-39</u>
Y	Federation Proceedings, Volume 44, Number 6, issued 12 March 1985 (Bethesda, Maryland, USA), R.K. Ito, "Fibrinolysis Studies: Fibrinogen-Specific Antibody As Carriers For Fibrinolytic Agents", see Abstract 8392.	27-30

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,147,765 (Stephan) 03 April 1979, see column 2, lines 42-44 and 55-63.	4-7,9-13, 15-18,23-37 39
X Y	Radiology, Volume 156, Number 2, 515-517, issued 1985 (Easton, Pennsylvania, USA), S.F. Rosebrough et al, "Radioimmunoimaging of Venous Thrombi Using Iodine-131 Monoclonal Antibody", see page 515, lines 22-27, page 516, column 2, lines 8-18 and page 517, column 2, lines 19-25.	1,5-9, 19-22 <u>10,23-39</u>

VI. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VII. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-18, 23-27 and 31-39 drawn to a monoclonal antibody, hybridoma and assay classified in Class 435 subclass 7. (Continued)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. **Telephone Practice.**
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

PCT/US87/02125

Attachment to Form PCT/ISA0/210, Part VI

II. Claims 19-22 drawn to a monoclonal antibody tagged with an imaging compound and the in vivo imaging procedure classified in Class 424, Subclass 9.

III. Claims 27-30 drawn to a monoclonal antibody-drug conjugate and its use for patient treatment classified in Class 424, Subclass 85.

PCT/US87/02125

Attachment to Form PCT/ISA/210, Part VI. 1.

Telephone approval:

\$280.00 payment approved by S. Leslie Misrock on 23 October 1987 for Groups II and III; charge to Deposit Account No. 16-1150.

Counsel advised that he has no right to protest for any Group not paid for and that any protest must be filed no later than 15 days from the date of mailing of the search report (Form 210).

Reasons for holding lack of unity of inventions:

The invention lack unity under PCT Rule 13 because the invention as defined by Group I (claims 1-18, 23-27 and 31-39) is drawn to a monoclonal antibody, hybridoma and assay which is classified in Class 435, Subclass 7 and which can be used in a process other than the process of Group II (claims 19-22) which is drawn to a monoclonal antibody tagged with an imaging compound and the in vivo imaging procedure classified in Class 424 Subclass 9 and which can be used in a process other than that of Group III (claims 27-30) which is drawn to a monoclonal antibody-drug conjugate and its use for patient treatment classified in Class 424, Subclass 85. For example the monoclonal antibody of Group I could be used for affinity chromatography.

Time Limit for Filing A Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack of unity only with respect to group(s) paid for.